Direct detection of mutations in the breast and ovarian cancer susceptibility gene BRCA1 by PCR-mediated site-directed mutagenesis

Elizabeth M. Rohlfs,1 William G. Learning,1 Kenneth J. Friedman,2 Fergus J. Couch,3 Barbara L. Weber,3,4 and Lawrence M. Silverman1*

The tumor suppressor genes BRCA1 and BRCA2, which confer increased susceptibility to breast and (or) ovarian cancer, were recently identified. Mutation analysis of BRCA1 has demonstrated significant allelic heterogeneity; however, some distinct mutations have been detected in unrelated individuals. The most notable is the 185delAG mutation, which occurs at an estimated frequency of ~1% in individuals of Ashkenazi Jewish descent [1]. Although consensus has not been reached regarding clinical testing for mutations in BRCA1, a tiered strategy may be appropriate, in which direct testing for the more common mutations is one component. Specific alleles can be detected by using PCR-mediated site-directed mutagenesis (PSM), which alters the PCR products derived from either the wild-type or mutant allele to create or destroy a restriction endonuclease recognition site. Recognition sites are introduced by a base substitution in one of the primers. The alleles are then resolved by electrophoresis of the digested PCR products. We have applied this technique to the detection of four BRCA1 mutations: 185delAG, 5382insC, E1250X, and R1443X. Another mutation, 1294del40, can be resolved from the wild-type allele by high-resolution gel electrophoresis alone. The PSM technique is sensitive, does not require radioactivity, and is specific for individual mutations.

INDEXING TERMS: polymerase chain reaction • ovarian cancer • genetics

1 Department of Pathology and Laboratory Medicine, CB7600, and 2 Curriculum in Genetics, University of North Carolina, Chapel Hill, NC 27599.
3 Department of Medicine, Room 1009, 422 Curie Blvd., and 4 Department of Genetics, University of Pennsylvania, Philadelphia, PA 19104.
*Author for correspondence. Fax 919-966-4526; e-mail lmsmol.dhl1@mail.unc.unc.edu.

Received June 18, 1996; revised August 16, 1996; accepted August 18, 1996.

Breast cancer afflicts >180 000 women in the US every year, and 5–10% of these cases are estimated to result from an inherited mutation in one of at least two genes [2]. The BRCA1 gene was linked to chromosome 17q21 in 1990 [3] and was isolated in October 1994 [4]. About half of the early-onset breast cancer families, and 80% of the early-onset breast and ovarian cancer families, are thought to reflect mutations in BRCA1 [5]. Women with germline mutations in BRCA1 have an estimated lifetime risk for breast cancer of 85% and a 20–50% risk for ovarian cancer [6]. Moreover, individuals who carry a BRCA1 mutation apparently have a fourfold increased risk for colon cancer, and carrier men have a threefold increased risk for prostate cancer [6].

BRCA1 is thought to be a tumor suppressor gene that encodes a protein capable of negatively regulating tumor growth. The gene spans >100 000 bp and has 22 coding exons. One exon (exon 11) accounts for >60% of the coding sequence. Mutations are distributed throughout the coding sequence with no apparent clustering or hot spots [7]. About 70% of the distinct mutations identified to date are small deletions or insertions that cause a frameshift in the coding sequence of the gene and result in a premature stop codon. Including nonsense mutations, ~86% of the distinct mutations detected so far are predicted to result in a truncated or shortened protein because of a premature termination of translation [8]. The function of the protein product is unknown, but it contains a RING finger motif thought to be involved in protein–protein interaction or nucleic acid binding (i.e., a transcription factor). Recently, the protein product has also been shown to contain a granin motif and may be secreted from the cell [9].

Analysis of BRCA1 in individuals with a strong family history suggestive of inherited disease has identified recurrent mutations in unrelated individuals as well as mutations that appear to be limited to particular families. More than 100 different mutations have been identified to
The 185delAG mutation has been detected in multiple individuals, most of whom are of Ashkenazi Jewish descent [1, 8, 10]. Among unrelated members of this ethnic group, the 185delAG mutation has been found on the same haplotype, indicating a likely founder effect [10-13]. However, other BRCA1 mutations, such as R1443X and 4184del4, have been found on different haplotypes—suggesting either de novo occurrence of these mutations in distinct populations or significant recombination near or within the BRCA1 gene [11, 12].

Given the large number of distinct mutations identified, many of which have been found only once, development of a protocol for mutation detection should include both mutation scanning methods and methods that detect specific mutations. Recurrent BRCA1 mutations have been detected by direct methods such as allele-specific oligonucleotide hybridization [1, 14] and allele-specific polymerase chain reaction (PCR) [15]. PCR-mediated site-directed mutagenesis (PSM) [16] identifies specific alleles by altering a sequence to introduce or remove a restriction site. The sequence is modified by substituting (or mismatching) a base near the mutation of interest in one of the two PCR primers. Thus, amplification of the region containing the mutation site will result in incorporation of the base change in the PCR product. After digestion with the appropriate restriction enzyme and electrophoretic separation of the fragments, the alleles can be identified. This technique is amenable to the detection of small insertions, deletions, nonsense and missense mutations and has been utilized for analysis of several genes, including those for cystic fibrosis transmembrane conductance regulator (CFTR) [17], low-density lipoprotein receptor [18], and phenylalanine hydroxylase [19].

**Materials and Methods**

**Patients’ Samples**

Samples were obtained from families at high risk for breast and (or) ovarian cancer, with three or more individuals with early-onset breast cancer or two with breast cancer and one with ovarian cancer. The mutations had been previously characterized by single-strand conformation polymorphism analysis or by conformation-sensitive gel electrophoresis and had been confirmed by sequencing. The K562 human cell line (chronic myelogenous leukemia, from ATCC, Rockville, MD) was used as a negative control.

DNA was extracted either directly from peripheral blood lymphocytes or from Epstein–Barr virus-transformed lymphoblastoid cell lines by using standard methods or the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer’s directions.

**PCR**

Primers were designed to either introduce or destroy a restriction site in either the wild-type or the mutant allele. To do this, we mismatched, or substituted, a base in the sequence of a primer that anneals adjacent to the mutation locus; this way, we could generate products with the corresponding base change (Fig. 1). The primer sequences used to detect each mutation are shown in Table 1. The 50-μL PCR reactions contained 10 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 2.0 mmol/L MgCl₂, 1.5 U of Taq polymerase (Perkin-Elmer, Norwalk, CT), 1 μmol/L each of the forward and reverse primers, 200 μmol/L deoxynucleotides, and 100 ng of template DNA. The amplification conditions were 95 °C for 2 min followed by 35 cycles of: 30 s at 95 °C, 30 s at 62.5 °C, and 1.0 min at 72 °C.

![Fig. 1. Primer design for mutation detection.](image)
The reactions were ended with a 10-min incubation at 72 °C. We used a Perkin-Elmer 9600 thermal cycler for all amplification reactions.

**RESTRICTION ENDONUCLEASE DIGESTION**

After the amplification, the PCR products were digested with the appropriate restriction endonucleases (New England Biolabs, Beverly, MA). The digestion reactions contained: 25.5 µL of PCR product, 3.0 µL of buffer (supplied with the enzyme), and 1.5 µL of enzyme (10 U). These components were incubated overnight at 37 °C. After 6 µL of 5× loading buffer (i.e., 300 mL/L glycerol and 10 g/L bromphenol blue in 10 mmol/L Tris, pH 8.0, plus 1 mmol/L disodium EDTA) was added to the digestion reaction, 15 µL of the digest was loaded on a 10% nondenaturing polyacrylamide gel and electrophoresed for 2 h at 325 V. The gel was stained with ethidium bromide reagent (0.5 mg/L in 134 mmol/L Tris, pH 8.6, containing 80 mmol/L boric acid and 30 mmol/L disodium EDTA) and transilluminated with ultraviolet light to visualize the bands.

**Results**

Amplification and digestion of PCR products spanning the 185delAG mutation are shown in Fig. 2. Both the 185delAG allele and the wild-type allele are normally cut by DdeI because the deleted AG is either preceded or followed by another AG, thus preserving the restriction site. However, when the C at nucleotide 188 of the noncoding strand is changed to a T by the mismatched reverse primer, the cutting site is destroyed in the mutant allele. Therefore, PCR amplification, followed by restriction enzyme digestion, produces fragments of 150 and 26 bp from the wild-type allele and an uncut fragment of 176 bp from the mutant allele (Table 2). The 26-bp fragment migrates quickly and is not seen on the gel. The pairing of single-strand PCR products, one of which contains the 2-bp deletion, results in the formation of two heteroduplex molecules [20]. The heteroduplex molecules have electrophoretic mobilities different from those of the homoduplex molecules and migrate above the 176-bp fragment, at ~181 and 189 bp.

Using the same mismatch primer strategy for 5382insC, introducing an A into the sequence at nucleotide 5384 destroys a BstNI site in the wild-type allele. The restriction site is still present in the mutant allele even with the mismatched base because the mutant C is inserted into a string of 3 Cs. Digestion of the mutant product results in 250- and 23-bp fragments (Fig. 2). Visible in the heterozygote sample are both the digested fragment of 250 bp from

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Exon</th>
<th>Primer(^a)</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>185delAG</td>
<td>2</td>
<td>F101-68</td>
<td>AAA ATG AAG TTG TCA TTT TAT AAA CC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R199 + 9</td>
<td>CTC ACT TAC CAG ATG GGA CAT T</td>
</tr>
<tr>
<td>5382insC</td>
<td>20</td>
<td>F5364</td>
<td>CCA AAG CGA AGA GAA TCA C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R5396 + 240</td>
<td>GAC GGG AAT CCA AAT TAC ACA G</td>
</tr>
<tr>
<td>E1250X</td>
<td>11</td>
<td>F3570</td>
<td>GAT GAC CTG TTA GAT GAT GGT GA</td>
</tr>
<tr>
<td>R1443X</td>
<td>11</td>
<td>R3890</td>
<td>CTC TCT GTI CTT AGA CAG ACC CT</td>
</tr>
<tr>
<td>1294del40</td>
<td>11</td>
<td>R4476 + 145</td>
<td>TCT TCT GCC CTT AGA GAC ATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F1178</td>
<td>GGA TAA AGA GAA ACT GCC ATG C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R1478</td>
<td>CTC TAC TGA TTT GGA GTG AAC TC</td>
</tr>
</tbody>
</table>

\(^a\) Mismatched bases are underlined.

\(^b\) Primers are designed according to their 5' nucleotide. F indicates forward and R indicates reverse.

---

**Fig. 2.** PSM detection of mutations in BRCA1.

After normal and mutant alleles were amplified by PCR, the products were digested with the appropriate restriction endonuclease and separated by electrophoresis as described in the text. For all gels: lane M, HaeIII-digested φX174 DNA size markers in basepairs; lane 1, individual heterozygous for the indicated mutation; lane 2, negative control; and lane 3, water blank.
the wild-type allele and the full-size product of 273 bp from the wild-type allele.

The nonsense mutation E1250X results from a G→T transversion at nucleotide 3867 and changes a Glu to a stop codon [8, 21]. This base change creates a natural cutting site for RsaI. However, because this enzyme has not been cloned and is therefore relatively costly, we introduced a StyI site into the mutant PCR product. The StyI site is introduced by using the reverse primer to mismatch the A at nucleotide 3870 of the anti-sense strand with a C. The full-length PCR product is 321 bp and its presence indicates the wild-type allele, whereas amplification and digestion of the mutant allele yields fragments of 300 and 21 bp (Fig. 2). Again, the heterozygote sample shows both fragments.

The R1443X mutation results from a C→T base transition at nucleotide 4446 and changes an Arg to a stop codon [21]. Introduction of an AlfIII site into the PCR product from the mutant allele results in fragments 180 and 17 bp long after restriction digestion (Fig. 1). The wild-type allele product is not digested but stays at 197 bp (Fig. 2). Another base substitution (C→G) at nucleotide 4446 has also been reported [8, 21] but is a benign polymorphism. This base change will not be detected by digestion with AlfIII, and the analysis will still indicate the presence of a wild-type allele. Alternatively, the sensitivity of PSM at this locus can be increased by designing the assay to produce a BsiUII cutting site in the wild-type allele. With this approach, virtually any base change at that particular locus will prevent the creation of the restriction site, and the analysis will indicate a non-wild-type allele. Although this design is more sensitive (i.e., can detect more base changes), it is not specific for a particular mutation.

A 40-bp deletion beginning at nucleotide 1294 can be detected by PCR amplification across the deletion and subsequent high-resolution gel electrophoresis. Our primer set amplifies a PCR product of 300 bp from the wild-type allele and a product of 260 bp from the mutant allele. Amplification of DNA from an individual heterozygous for this mutation produces the two expected bands as well as a heteroduplex band that migrates significantly more slowly (at ~2300 bp) than either homoduplex molecule (Fig. 2).

**Table 2. PSM restriction fragments.**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Enzyme</th>
<th>Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>185delAG</td>
<td>Ddel</td>
<td>150, 26, 176, 181, 189</td>
</tr>
<tr>
<td>5382insC</td>
<td>BstII</td>
<td>273, 250, 23</td>
</tr>
<tr>
<td>E1250X</td>
<td>StyI</td>
<td>321, 300, 21</td>
</tr>
<tr>
<td>R1443X</td>
<td>AlfII</td>
<td>197, 180, 17</td>
</tr>
<tr>
<td>1294del40</td>
<td>None</td>
<td>300, 260, –2300</td>
</tr>
</tbody>
</table>

*Discussion*

Except for a few disorders such as sickle cell anemia and α1-antitrypsin deficiency, most single-gene disorders are characterized by significant allelic heterogeneity. More than 500 different mutations that cause cystic fibrosis have been identified in the CFTR gene, and >170 different mutations that cause phenylketonuria have been identified in the phenylalanine hydroxylase gene [22, 23]. In addition, many of these mutations are limited to individual families. At present, we do not yet know how many different mutations will be identified in BRCA1, and a comprehensive protocol for mutation detection is thus difficult to formulate. Methods such as single-strand conformation polymorphism, denaturing gradient-gel electrophoresis, or the protein truncation test have proven useful for scanning coding regions and critical intron sequences for sequence variants in a variety of disorders [24-26]. After screening large numbers of individuals, investigators have found that, among unrelated individuals with some disorders, certain mutant alleles are found more frequently than others. These recurrent alleles may be detected by using direct PCR-based methods such as allele-specific oligonucleotide hybridization [1, 14], allele-specific PCR [15], or PSM [17].

We have applied PSM to the detection of four mutations found in BRCA1. This technique is suitable for detecting single-base changes, small deletions, and small insertions. Because detection is based on alteration of restriction endonuclease recognition sites, PSM is highly specific for the mutation of interest when the assay is designed to cut the mutant allele. For this reason, the PCR products from the mutant alleles E1250X, 5382insC, and R1443X contain the restriction sites. When a constitutive cutting site is present within the PCR product, the analytical sensitivity of the assay is increased because the constitutive site acts as a control for enzyme activity.

Designing a PSM for 185delAG was somewhat more difficult because of the repetitive AG and thus required digestion of the wild-type allele. Therefore, any alteration in the sequence that disrupted the DdeI recognition site would prevent digestion and indicate a non-wild-type allele. In this respect, the assay became more sensitive but less specific.

PSM analysis can also be affected when a mutation other than the one in question happens to alter the region where a primer should anneal. For example, there have been several reports of an 11-bp deletion in BRCA1 beginning at nucleotide 188 [4, 8, 13]. This mutation will prevent the 185delAG reverse primer from annealing and thus will prevent amplification from the mutant allele; the results will indicate the presence of only a wild-type allele. Also, a 4-bp deletion within the E1250X reverse primer binding site may prevent amplification or indicate a heteroduplex formation if amplification does occur. To the extent that confounding mutations are subsequently found to be moderately prevalent, PSM assays may be readily designed for these mutations.
The combination of PSM and high-resolution gel electrophoresis allows us to detect five BRCA1 mutations, two of which (185delAG and 5382insC) are the mutations detected most frequently (Table 3) [8]. R1443X and 1294del40 are also recurrent mutations but at a lesser frequency, and E1250X has been identified only twice [8,21]. As an allele-specific detection technique, PSM offers many advantages over other methods. It does not require the use of radioisotopes, it is relatively cost effective, and it does not require a subjective evaluation—as sometimes occurs with oligonucleotide hybridization methods. In addition, PSM is extremely flexible. With >150 restriction enzymes commercially available, almost all single-base changes and small deletions and insertions can be unequivocally identified.

Use of a direct test, such as PSM, to detect the more common mutations may be a useful component of a tiered testing program (Fig. 3). Initial screening of a patient’s sample for the five mutations described here would detect ~28% of the characterized BRCA1 alleles [8]. If no mutation was detected by PSM, a gene-scanning technique could be used to screen for less common, private mutations. The scanning technique chosen would depend on the types of mutations that occur in the gene (i.e., protein truncation test to detect truncating mutations, heteroduplex analysis to detect missense mutations). Any putative mutations identified by the scanning techniques would be confirmed by sequencing.

The technical aspects of mutation detection have now advanced sufficiently to permit analysis of any portion of the human genome. We strongly recommend judicious use of detection strategies, such as those described here, especially when analyzing human DNA for mutations that may predispose an individual to an adult-onset disorder. Although it is not within the scope of this paper to discuss all the relevant social (i.e., insurance and employment discrimination) and scientific (i.e., penetrance of particular alleles, polymorphism vs mutation) issues involved with clinical genetic testing, we recommend to the reader several recent articles that discuss these topics [27,28].

This work was supported in part by National Institutes of Health grant NRSA CA62588 to F.J.C.

References

12. Neuhausen SL, Mazoyer S, Friedman L, Stratton M, Offit K, Caligo...


